"Optimization of Methyl Red Dye Decolorization by *Staphylococcus aureus* and Toxicity Assessment via Seed Germination Bioassay"

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

Submitted By
PAULAMI CHAKRABORTY
Registration ID: 230010426122
Enrollment ID: 2322211001042

Advisor
Ms. Pranati Das
Research and Training Co-ordinator
SHRM Biotechnologies Pvt Ltd
Kolkata

School of Health Science and Translational Research
Sister Nivedita University
Kolkata
June 2025

SHRM Bio-technologies Pvt Ltd
CINNO–U40107WB2006PTC110903 MSMENo–WB14D0013023
Honorary Recognization From LSSSDC, NSDC, Govt. of India. An ISO 9001:2015 Certified Organisation

Humaipur. PO: Abdalpur
Madhyamgram Kolkata 700155
Phone: +918981003215
Email:info@shrmbio.com
www.shrmbio.com

Ref:-SHRM/WB/1404-06/ Date:-30/06/2025

CERTIFICATE

This is to certify that ~~Mr.~~/ Miss **Paulami Chakraborty**, student of Sister Nivedita University Reg. No.- 230010426122 has completed the 6 months Project work, entitled "***Optimization of Methyl Red Dye Decolorization by Staphylococcus aureus and Toxicity Assessment via Seed Germination Bioassay***", under the guidance of **Pranati Das** at **SHRM Biotechnologies Pvt. Ltd.**, Madhyamgram, West Bengal.

We wish her all the best for her future endeavors.

As a student she has proved herself diligent, sincere and well behaved.

SHRM BIOTECHNOLOGIES PVT LTD KOLKATA

Pranati Das 30/6/25

Mr. Kunal Vora
CEO & Director
SHRM Biotech

Pranati Das
Project Co-ordinator
SHRM Biotech

Awarded the Best Biotech Training provider in Eastern India- 2018 by Union Minister of Agriculture AwardedTrainingExcellenceAwardonLifeSciencesbyHon'ble governorsofOrissa&Tripurain2017

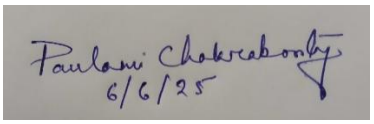
DECLARATION BY STUDENT

I hereby declare that the work which is being presented in this dissertation entitled **"Optimization of Methyl Red Dye Decolorization by *Staphylococcus aureus* and Toxicity Assessment via Seed Germination Bioassay "** in the partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from December to May under the supervision of **Ms. Pranati Das**, **Research and training coordinator,** at **SHRM BIOTECHNOLOGIES PVT LTD, MADHYAMGRAM**.

I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions*).

I confirm that this dissertation has been checked for plagiarism using the plagiarism checking software-Small SEO tools. At the end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential source (if any), generated online using plagiarism checking software is enclosed. I herewith confirm that the dissertation has been less than 10% similarity according to the plagiarism checking software's report and meets the UGC Regulations as well as plagiarism rules of Sister Nivedita University.

I further declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and findings described in my dissertations.



(Signature of the student with Date)

PAULAMI CHAKRABORTY

REGISTRATION NO 230010426122

MSc. MICROBIOLOGY (2023-2025)

SISTER NIVEDITA UNIVERSITY
সিস্টার নিবেদিতা বিশ্ববিদ্যালয়
DG Block - 1/2, Action Area I, New Town, Kolkata - 700156, West Bengal, India.

DEPARTMENT OF BIOTECHNOLOGY & DEPARTMENT OF MICROBIOLOGY
জৈব প্রযুক্তি বিভাগ ও অণুজীব বিদ্যা বিভাগ

TO WHOM IT MAY CONCERN

This is to certify that the project report titled " **Optimization of Methyl Red Dye Decolorization by *Staphylococcus aureus* and Toxicity Assessment via Seed Germination Bioassay** ", submitted by Mr./Ms. **Paulami Chakraborty** (Registration No. 230010426122 ,Enrolment No. 2322211001042) to the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by him/her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 6.06.2025

Place: Kolkata

Signature of Head of the Department
Seal

ACKNOWLEDGMENT

I, Paulami Chakraborty would like to express my sincere gratitude to all those who supported and guided me throughout the completion of this project.

First and foremost, I would like to thank Ms. Pranati Das, Research and Training Co-Ordinator, SHRM Biotechnologies Pvt Ltd, for their invaluable guidance, encouragement, and support throughout the duration of this work.

I am also thankful to Mr. Kunal Vora, Dr. Rupak Roy and Ms. Shreosee Ghosh for providing the necessary resources and a conducive environment for this work. Their expertise and insights greatly helped shape this project.

I extend my heartfelt thanks to all the faculty members and lab staff of the SHRM Biotechnologies Pvt ltd, whose assistance and cooperation have been instrumental.

Finally, I am deeply grateful to my family and friends for their constant motivation, patience, and encouragement during this project.

PAULAMI CHAKRABORTY
ENROLLMENT NO. - 2322211001042
MSC MICROBIOLOGY (2023-2025)

LIST OF FIGURES

FIGURE NO.	DESCRIPTION	PAGE NO.
1	Core structure of an Azo dye	10
2	Dye remediation methods comparison	12
3	Microbial degradation processes	13
4	Serial dilution of the collected soil	39
5	Spreading on nutrient agar treated with Methyl Red	40
6	Sample streaking on nutrient agar plate containing Methyl Red	40
7	Master plate	41
8	Streaking on MSM agar containing only carbon source	41
9	Gram staining	42
10	Indole test	43
11	Methyl Red test	43
12	Voges-Proskauer test	44
13	Citrate test	44
14	Starch Hydrolysis test	45
15	Urease test	45
16	Gelatinase test	46
17	Catalase test	46
18	Oxidase test	47
19a	Sugar fermentation test	47
19b	Dextrose test	48
19c	Lactose test	48
19d	Sucrose test	48
19e	Sorbitol test	48
19f	Maltose test	48
20	MSA test	49
21	OD MSM broth taken at 428.87nm at different time intervals	50
22	pH effect on the degradation efficiency of Methyl Red dye	51
23	Temperature effect on the degradation efficiency of Methyl Red dye	53
24	Salinity effect on the degradation efficiency of Methyl Red dye	55
25	Seeds treated with (a) dye solution at 60˚C, (b) dye solution with 0.1gm salinity, (c) basic dye solution, (d) untreated dye solution, (e) water	57
26	Seed germinated after 7 days (a) dye solution treated at 60˚C, (b) dye solution with 0.1 gm salinity, (c) basic dye solution, (d) untreated dye solution, (e) water	58

LIST OF TABLES

TABLE NO.	DESCRIPTION	PAGE NO.
1	Media composition for MSM	19
2	Principle for IMVIC test	21
3	Media composition for Indole test	22
4	Media composition of MR-VP broth	24
5	Media composition for Citrate test	25
6	Media composition for Strach Hydrolysis	26
7	Media composition for Urease test	27
8	Media composition of Gelatinase test	29
9	Media composition for Sugar test	31
10	Media composition for MSA	33

ABBREVIATIONS

- TCA cycle: Tricarboxylic Acid Cycle
- NADH: Nicotinamide adenine dinucleotide-hydrogen
- $FADH_2$: Flavin Adenine Dinucleotide
- GMO: Genetically Modified Organisms
- MSM: Minimal Salt Medium
- MR test: Methyl Red test
- VP test: Voges-Proskauer test
- MSA: Mannitol Salt Agar
- OD: Optical Density

ABSTARCT

Synthetic dyes are extensively utilized in the textile industry due to their desirable chromatic properties and chemical stability; however, their release into the environment poses substantial ecological risks. Owing to their complex aromatic structures, these compounds exhibit high resistance to natural degradation, leading to prolonged environmental persistence. This study aimed to evaluate the detoxification efficiency and residual toxicity of synthetic dye solutions subjected to various physico-chemical treatments, including elevated temperature (60 °C), increased salinity (0.005, 0.01, 0.1 g/L NaCl), and altered pH (acidic and basic conditions). The extent of dye degradation and associated biological impact were assessed via a seed germination bioassay using Vigna radiata as a model organism.

Spectrophotometric measurements at 428.87 nm over a 4-hour period tracked optical density changes in dye solutions. Degradation was most pronounced in the thermally treated dye at 60°C, showing a significant decrease in optical density. Treatments with basic pH and high salinity also induced moderate degradation, while the untreated dye remained stable, indicating resistance to natural breakdown. Bioassay results corroborated these findings, with seeds exposed to the thermally treated dye exhibiting high germination and root elongation rates, similar to controls, while seeds exposed to the untreated dye showed significantly reduced germination and elongation, suggesting toxicity. Salinity and pH treatments had intermediate biological effects

The findings demonstrate that thermal and chemical treatments can modify the chemical structure of dyes, potentially reducing their persistence and toxicity. The observed decrease in optical density reflects chromophore degradation, which likely lowers toxicity, as simpler residual compounds tend to be less biologically harmful.

This study highlights the promise of simple, cost-effective strategies for mitigating dye toxicity. The results provide a practical framework for industries and environmental agencies to adopt more eco-friendly and scalable pollution control measures.

Keywords: Synthetic dyes, Dye degradation, Temperature treatment, Salinity, pH, Seed germination assay, Toxicity, Environmentally friendly

CONTENTS

S. NO.	DESCRIPTION	PAGE NO.
1	INTRODUCTION	10
2	REVIEW OF LITERATURE	13
3	MATERIALS AND METHODS	17
4	RESULTS AND DISCUSSION	39
5	CONCLUSION	60
6	APPENDIX	61
7	REFERENCES	62
8	PLAGIARISM REPORT	65

INTRODUCTION

Dyes are natural and synthetic substances that are widely used in many consumer products in a variety of industrial sectors, including pharmaceuticals, food and paper industries, but most commonly in the textile industries (Sharma et al.,2016). There are several types of dyes that are used which can be acidic, basic, reactive, direct dyes depending on the structures. The most popular industrial dyes include azo dyes, indigo dyes, sulfur dyes etc. (Shahid et al., 2013). The large-scale use of these dyes in the textile industries due to their diversity in structure, low energy consumption, cost effectiveness, large range of color varieties and ease of applications. A variety of chemicals are used to constitute a vast array of dye types in production, varying based on the textile or product dyed.

Azo dyes account for the largest group of dyes being used in the industries currently. Azo compounds constitute diverse group of synthetic dyes and are the widely used in the industries. Due to the strong interactions of dyes with water, these compounds are used in aqueous solutions. The chromophore part in azo dyes provide bright colors and thus used extensively in textile, leather and food industries. The central structure of these dyes is an azo group (-N=N-), where two nitrogen atoms are double-bonded to each other (Hunger, 2003).

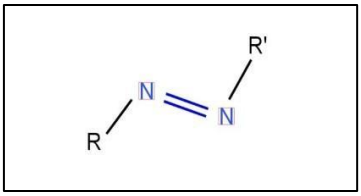


Fig 1: Core structure of an Azo dye

Azo dyes are divided into three major types based on the number of azo groups. These are mono-azo dyes containing one azo group, diazo dyes containing two azo groups and poly-azo dyes containing more than two azo groups. These groups are attached to either an aromatic or heterocyclic compound on one side and contain an unsaturated heterocycle, carboxyl, sulphonyl or aliphatic group on the other side. Methyl red is such an anionic mono-azo dye. It is a common pH indicator that turns red in acidic solutions. The textile industries are believed to generate 80% of azo dyes of synthetic origin for various dyeing purposes where It is estimated that during these processes approximately 10-15% about the total dye made is not absorbed by the fibers during processing and therefore the discharged effluent requires remediation (Pirkarami and Olya, 2017). The stability and persistence of the azo dyes leads to the pollution of the environment. Some of these dyes have detrimental effects not only on the environment but also on human health due to their toxicity and persistence (Benkhaya et al.,

2020).

The textile industries nowadays use large amount of these dyes and chemicals, these industrial effluents are major contributor to water and soil pollution. Studies indicate that the dangers of releasing untreated azo dyes into the environment are primarily related to and are a result of the aromatic amines that are the products of cleavage of the azo groups (-N=N-) (Pinheiro et al., 2004). These, which carry a high-level risk to human, animals as well as plants and the overall environment, have been characterized as important mutagens and carcinogens (Holme, 1984). Each dye's biological and toxicological activities can vary dramatically, even among the dyes within the same group and with the same chemical structure (Chen et al., 2018). Even in low concentration, azo dyes cause detrimental effects on ecosystems. Dumping untreated dyes without treatment directly into the soil, water bodies not only effect the nature of the soil or water but also cause risk to the living organisms living in it. Dispersion of these azo dyes through the environment affects more of such living organisms in surrounding and this could also pose harmful impacts on the growth and seed germination of plants. Dye waste effluents discharged into the waterways and waterbodies limits the ability of light to travel and preventing aquatic plants from photosynthesizing (Elumalai and Saravanan, 2016). Improper disposal leads to accumulation of heavy metals and chemicals in soil, thereby making soil unproductive and limiting plant life. These toxic compounds may enter the food chain, accumulating in higher organisms, including humans. The discharge of dye affluents from textile industries has some adverse effect on human health causing dermatitis, allergies, eye irritation. Inhalation of volatile compounds can also cause respiratory issues (Chung, 2016). Some of the dyes are known to release carcinogenic amines. Therefore, it is very crucial to treat industrial dye effluents before being disposed to avoid such damage to the environment as well as to the living beings. In recent years different treatment techniques have been used by the researchers to remove these azo dyes from effluents for a sustainable environment.

There are a variety of physical and chemical methods that has been employed which include filtration, adsorption, precipitation, sonication, photolysis, chemical oxidation, coagulation, electrolysis (Malik et al., 2017, Rawat et al., 2018), to remediate dye effluents. However, these methods have significant drawbacks such as low versatility, low efficiency, high cost etc (Chen et al., 2018). Moreover, due to the persistent nature and chemical instability of the azo dyes, it has been found that some of the treatment techniques are not effective. Ongoing researches are being conducted to identify an effective solution to this issue. Recent studies have focused on

utilizing microorganisms to degrade these dye effluents diffused from the industries, aiming to mitigate their harmful impacts which are more cost-effective and environment friendly. Microorganisms' capacity for decolorization and degradation of dyes has attracted a great deal of attention (Naraginti et al., 2016).

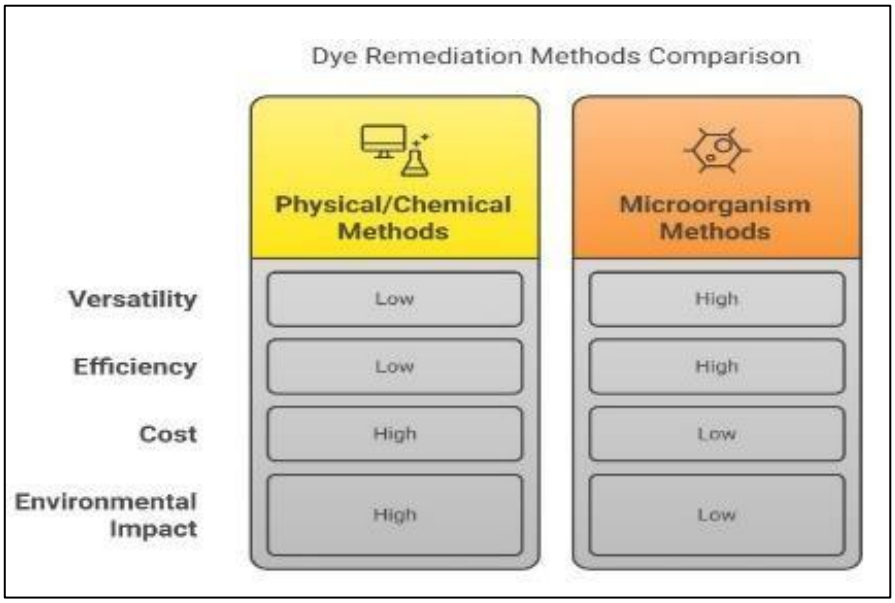


Fig 2: Dye remediation methods comparison

The basic aspects to decolourize and degrade a dye is to break down the azo bond. Many bacterial strains can transform many of these toxic compounds to non-toxic by products. Diverse bacterial species can completely mineralize many azo dyes. The breakdown of azo dyes by bacteria typically occurs in two main stages. Initially, the azo bonds within the dye molecules are cleaved by the action of the enzyme azoreductase secreted by the bacteria, leading to the production of aromatic amines (Balapure et al., 2014). These intermediate compounds are known to be toxic. In the subsequent stage, these aromatic amines undergo further degradation into less harmful substances or are fully mineralized (Garg and Tripathi, 2017), thereby reducing their environmental impact. As the azo dyes undergo degradation, a corresponding decolourization of the dye molecule can also be observed, indicating structural disruption and loss of conjugated systems associated with their chromophoric properties.

Although there are only a few studies currently available on the degradation of methyl red, especially using *Staphylococcus* sp., this study aims to evaluate the degradation potential and elucidate the degradation mechanism of selected *Staphylococcus* sp. strain. Furthermore, the effects of various physicochemical parameters (pH, temperature, salinity etc) was carried out to maximize the efficiency and effectiveness of the degradation process of Methyl Red dye.

REVIEW OF LITERATURE

The textile sector is one of the main causes of environmental contamination, largely due to the high amounts of synthetic dyes in textile and dyeing production (Forgacs et al., 2004). Traditional methods of removing dye including chemical oxidation and physical adsorption, tend to leave residues behind and can cause secondary pollution (Robinson et al., 2001). Microbial degradation represents a more environment friendly and economical way to remediate dyes. In recent years, there has been a greater focus on assessing the causation behind environmental pollution, one of which is industrial discharge of dyes. due to the inherent toxicity of dyed, they pollute the environment. The Central Pollution Control Board has recognized the dyeing industry as a heavily polluting industry (Rajan 2004).

Microbial degradation of textile dyes involves complex biochemical pathways, in which microorganisms use dyes as carbon, nitrogen or energy source, or co-metabolize the dyes in combination with other substrates. The mechanisms depend on various factors, including the type of the dye, the species of microorganism and the environmental conditions (Pandey et al., 2007).

Microorganisms degrade dyes primarily through enzymatic catalysis, which involves:

- Reduction reactions
- Oxidative cleavage
- Hydrolytic degradation
- Biosorption and bioaccumulation

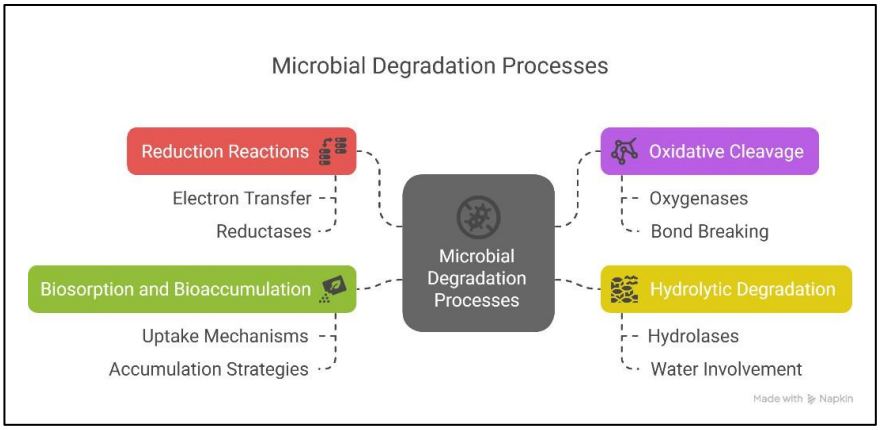


Fig 3: Microbial degradation processes

Some common steps of microbial degradation are as follows:

1. Adsorption: Dyes first adhere to the cell wall or surface of the microbe.
2. Enzymatic attack: The microbe produces specific enzymes that hydrolyse the bonds of the dye molecule.
3. Metabolism: The dye fragments are assimilated to the TCA cycle and other central metabolic pathways for full degradation.
4. Mineralization: The end product of dye metabolism are CO_2, H_2O and inorganic ions are released.

The efficiency of microbial dye degradations is also highly dependent on several physicochemical factors, including temperature, pH, salinity, dye structure etc. Understanding and optimizing these parameters are crucial to achieve optimal dye degradation rates. The rates of the degradation can be assessed by quantifying optical density at a specific wavelength at regular intervals. To evaluate different physical factors, batch decolorization studies should be carried out under static culture conditions with varying temperatures, pH and salinity.

Microbial degradation of dyes can also be improved by providing appropriate co-substrates like carbon and nitrogen sources. Microbial growth and enzyme production in stimulated by various carbon sources like glucose, starch, lactose and other simple sugars. Ammonium salts, peptone are sources of nitrogen which support biomass production and metabolic activity. Many synthetic dyes are poor sources of carbon and energy. Supplementing with an external carbon source boosts microbial metabolism, supporting better growth and enzyme synthesis. When analysing the degradation of Methyl Red (a general azo dye), carbon sources are added separately for various fundamental scientific reasons:

Methyl red can itself act as a both nitrogen and carbon source for a certain microorganism. Providing an independent carbon source allows scientists to observe whether the bacteria is utilizing the dye mainly as a carbon source or simply cleaving the azo bond.

Several microorganisms require a supplemental carbon source in order to efficiently degrade methyl red. The carbon source is used to supply energy for the formation of enzymes used in the degradation of the dye.

For the degradation of methyl red, it is generally accomplished through co-metabolism. In this case the carbon source serves as the main substrate for microbial respiration. The dye is being co-metabolized as a secondary substrate. As such the carbon source does participate in the dye degradation but it does not serve as a substrate for the actual degradation of methyl red. The

carbon source provides the reducing equivalents, like NADH or $FADH_2$, necessary for the enzymes to break the azo bond of the dye.

The carbon source type and concentration have a significant influence on the degradation kinetics of methyl red. For instance, glucose and starch have been shown to enhance the rate of degradation, whereas too much carbon may lead to catabolite repression, where microbes tend to use the added carbon preferentially and inhibit dye degradation pathways. Therefore, optimization of the carbon source type and dosage is important for maximum efficiency.

Varying types and concentrations of carbon sources enables researchers to improve degradation conditions. This assists in identifying the most effective setup for industrial purposes.

Over the past few decades, a variety of physicochemical methods have been used to remove textile dyes from the effluents of the industries, such as coagulation-flocculation, chemical, oxidation, adsorption on activated carbon, photocatalysis (Robinson et al., 2001; Forgacs et al., 2004). Although these treatments will decolorize and remove dye from industrial effluents to significant levels, there are characteristically major drawbacks. For instance, some of these processes require high energy intakes and expensive reagents and can generate toxic secondary pollutants that require further treatment (Gupta & Suhas, 2009). Additionally, these traditional treatments fail to be effective against recalcitrant dye structures like polyazo, anthraquinone dyes which are resistant to oxidative degradation. Further, most of them are unsustainable in the sense that they fail to mineralize the dyes completely into innocuous end products, but instead deposit harmful aromatic amines and partially degraded intermediates (Forgacs et al., 2004).

These limitations have resulted in the increasing focus of research onto biological alternatives, specially, on microbial degradation, as a more environmentally friendly, cost-effective and sustainable alternative. Microorganisms have the enzymatic machinery to not only decolorize but also detoxify and mineralize a wide assortment of synthetic dyes under mild environmental conditions (Saratale et al., 2011).

Thus, research today focuses on isolating and optimizing dye-degrading bacteria, fungi, yeast and algal systems, as well as developing microbial consortia that can perform robust degradation across diverse industrial settings.

Despite the promising potential of microorganisms for textile dye degradation, several challenges still hinder the large-scale application. Maintaining the right conditions of

temperature, pH, salinity is challenging to implement at an industrial scale, affecting microbial efficiency (Saratale et al., 2011). Some microorganisms have narrow substrate range and degrade only specific dyes, while higher concentration of dyes and presence of heavy metals can deter microbial growth and decrease enzymatic activity (Forgacs et al., 2004). Although there have been a number of successful results from a laboratory perspective, transferring this success to real-world treatment plants is still challenging. Issues such as microbial instability, loss of the degradation capacity over time further complicate the consistency of microbial treatment systems. Researches are still actively ongoing to address the challenges associated with microbial dye degradation, with a focus on enhancing efficiency, stability and large-scale applicability.

To overcome current challenges, several future directions and innovations are being explored. Development of robust microbial consortia using mixed microbial cultures can enhance dye degradation. Also, genetic engineering now allows scientists to construct genetically modified organisms (GMO) and each genetically modified organism should be producing higher performance dye degrading enzymes, have large scale of substrate specificity, and heightened tolerance to toxic compounds. Immobilizing microbes on carriers like activated carbon can improve their operational stability, making industrial application more feasible (Ali, 2010).

As environmental issues become a global priority, future researches are focusing on developing affordable, scalable, and low-maintenance microbial systems that are suitable for use in developing regions with textile industries as important agents of economic growth. Continued research into the area of microbial density, genetic enhancement, and system optimization is necessary in order for the condition of microbial dye degradation to become a standard and eco-friendly practice in textile industries around the globe.

MATERIALS AND METHODS

1. Isolation of the bacterial strain:

- Sample Collection:

  Soil samples were collected from the area adjacent to a drainage system, approximately 10cm beneath the soil surface, using a sterile spatula. The collected soil was immediately transferred into sterile, labelled plastic bags and brought to the laboratory for further processing. Care was taken to avoid contamination during sample collection and transport.

- Serial dilution:

  In the laboratory, 1 gram of the soil sample was suspended in 10 ml of sterile distilled water in an autoclaved sterile test tube and mixed thoroughly to obtain a uniform suspension. Then a series of 10 folds serial dilutions were prepared in autoclaved sterile test tubes by transferring 1 ml of the suspension into 9 ml of sterile distilled water up to the dilution of 10^{-4}, using sterile pipettes and aseptic techniques in the laminar airflow.

- Spreading on agar plate:

  Nutrient agar medium was prepared following standard protocols and supplemented with 40 ppm Methyl Red dye after autoclaving. The medium was poured into the sterile petri dish under aseptic conditions and allowed to solidify. Once solidified, 0.1 ml aliquots from the dilution of 10^{-4} were pipetted onto the surface of Methyl Red containing nutrient agar plates. The aliquots were evenly spread using a sterile glass spreader. The plate was then left for a few minutes to absorb the inoculum, then incubated at 37˚C for 24 hours to allow visible colony development and dye degradation.

- Streaking for confirmation:

  After incubation, a well isolated colony was chosen from the plates. The colony was picked using a sterile inoculating loop and streaked onto a fresh nutrient agar plate supplemented with the 40 ppm Methyl Red dye using the simple streaking technique. The plate was incubated under the same conditions to

confirm that the dye was degraded by the bacterial strain.

- Preparation of master plate:
  After the confirmation that the bacterial strain was successfully degrading the dye, one single colony was picked from that previously streaked plate and streaked onto a fresh nutrient agar plate to create a uniform and abundant source of the isolate. This master plate was incubated overnight and subsequently used for further analysis.

2. Preliminary Decolourization:

To evaluate the decolourization potential of the isolated bacterial strain, a preliminary experiment was conducted using Minimal Salt Medium (MSM), supplemented individually with either a carbon source or a nitrogen source, along with the 40 ppm Methyl Red dye. This experiment aimed to evaluate which nutrient component supports more dye degradation by the isolate.

Preparation of Media:

Initially, 50 ml of MSM agar solution without adding any carbon or nitrogen source was prepared in a 100 ml clean conical flask. The medium was then equally divided into two separate conical flasks (25 ml each):

- To the first flask, a carbon source glucose (10 g/L) was added after autoclave and labelled.
- To the second flask, a nitrogen source Ammonium nitrate (0.1 g/L) was added and labelled.

Both media was subjected to autoclaving at 121℃ for 15 mins to ensure sterility. After autoclaving, both media was cooled down slightly under laminar airflow.

Addition of Dye and inoculation:

40 ppm dye solution was prepared and added aseptically to each flask and was mixed

thoroughly. Then the MSM agar, one with carbon source and one with nitrogen source was poured into different petri plates and was allowed to solidify. After solidification, each plate was inoculated with the test strain taken from master plate under sterile conditions.

Incubation:

Both the petri plates were then incubated in inverted position at 37 ˚C for 24 hours.

TABLE 1: Media composition for MSM

REAGENT	**STANDARD WEIGHT (S.W) FOR 1000 ml**	**CALCULATED WEIGHT (C.W) for 50ml**
Peptone	5 gm/L	0.25 gm
Sodium Chloride (NaCl)	0.1 gm/L	0.005 gm
Potassium hydrogen phosphate	4 gm/L	0.2 gm
Magnesium sulfate	0.1 gm/L	0.005 gm
Ammonium nitrate	0.1 gm/L	0.005 gm
Glucose	10 gm/L	0.5 gm
Agar	20 gm	1 gm
Distilled water	1000 ml	50 ml

3. Identification of isolated strain:

Characterization was done to identify and understand the morphological, biological and physiological properties of the microbial strain we got from the soil.

I. GRAM STAINING:

Gram staining is a differential staining technique that classifies bacteria into two major groups:

- Gram-positive bacteria: Retain the crystal violet stain and appear purple.
- Gram-negative bacteria: Do not retain the crystal violet but take up the counterstain (safranin) and appear pink/red.

This difference is due to the structure of the bacterial cell wall:

- Gram-positive bacteria have a thick peptidoglycan layer that traps the crystal violet-iodine complex.
- Gram-negative bacteria have a thin peptidoglycan layer and an outer membrane that allows the complex to be washed out by alcohol.

Materials Required

- Clean glass slide
- Bacterial culture (smear)
- Crystal violet (primary stain)
- Gram's iodine (mordant)
- 95% Ethanol or Acetone-alcohol (decolorizer)
- Safranin (counterstain)
- Distilled water
- Staining rack
- Bunsen burner
- Inoculating loop
- Microscope

Procedure:

- A small drop of distilled water was placed on a clean glass slide.
- A small amount of bacterial culture was transferred from the master plate with a sterile loop.
- It was Mixed and spreaded to make a thin smear.

- The smear was air dried and heat fixed by passing the slide over a flame 2–3 times.
- Staining Steps:

  Crystal Violet: The smear was flooded with crystal violet for 30 seconds and then rinsed with water.
  Gram's Iodine: Gram's iodine was added and left for 30 seconds, after that it was rinsed.
  Decolorizer (Ethanol/Alcohol): Ethanol was applied for 10–30 seconds (until runoff is colorless) and then was rinsed immediately.
  Safranin: Counterstain safranin was added and left for 30 second. Then it was rinsed and blot dried.
- Microscopy: The slide was examined under a microscope using oil immersion lens in 100x lens.
- The shape, color, size of bacteria was observed and an image of result was taken.

II. BIOCHEMICAL TEST:

➢ IMVIC TEST

TABLE 2: Principle for IMVIC Test

TEST	PRINCIPLE
Indole (I)	Detects the ability of bacteria to break down tryptophan to indole using the enzyme tryptophanase.
Methyl Red (MR)	Detects mixed acid fermentation of glucose, producing stable acidic end-products that lower the pH.
Voges-Proskauer (VP)	Detects acetoin formed during glucose fermentation via the butylene glycol pathway.
Citrate (C)	Tests whether an organism can use citrate as the sole carbon source and ammonium salt as the nitrogen source.

1. INDOLE TEST

Procedure:

- 20 ml of media solution was prepared in a conical flask.
- Prepared media was transferred into two test tubes, one for control and one for test strain.
- 2 test tubes (each containing 10 ml) were taken and cotton plugged and wrapped with paper or aluminium foil to set in autoclave in a beaker.
- After autoclave the media was inoculated:

  One sterile tryptone broth tube was taken (test sample) and was

  aseptically inoculated it with a loopful of the test organism taken from the master plate.
- Inoculated test tube along with the uninoculated control were incubated at 35–37°C for 24 hours.
- After incubation, 3–5 drops of Kovac's reagent were added to the surface of the broth.
- After a few minutes and the color of the interface was observed.

TABLE 3: Media composition for indole test

REAGENT	**STANDARD WEIGHT (S.W) FOR 1000 ml**	**CALCULATED WEIGHT (C.W) for 20ml**
Tryptone	10.0 gm/L	0.2 gm
Sodium Chloride (NaCl)	5.0 gm/L	0.1 gm
Distilled Water	1000 ml	20ml

2. METHYL-RED TEST

Procedure:

- 20 ml of media solution was prepared in a conical flask.
- 2 test tubes (each containing 10 ml) were taken and cotton plugged and wrapped with paper or aluminium foil to set in autoclave in a beaker.
- Prepared media was transferred to two test tubes, one for the test strain and one for control.
- Inoculated the Medium:

  One sterile test tube was taken and labelled as test strain, was aseptically inoculated with a loopful of the test organism taken from the master plate.
- Inoculated test tube along with the uninoculated control were incubated at 35–37°C for 24 hours.
- After incubation, 3–5 drops of Methyl Red Indicator was added to the surface of the broth and gently the tubes were shaken.
- After a few minutes the color change was observed immediately.

3. VOGES-PROSKAUER TEST

Procedure:

- 20 ml of VP Broth was prepared in a conical flask.
- 2 test tubes (each containing 10 ml) were taken and cotton plugged and wrapped with paper or aluminium foil to set in autoclave in a beaker.
- Prepared media will be transferred to two test tubes, one for test sample and one for control.
- Inoculated the Medium:

  One sterile test tube was taken and labelled as test strain, was aseptically inoculated with a loopful of the test organism taken from the master plate.
- Inoculated test tube along with the uninoculated control were incubated at 35–37°C for 24 hours.
- After incubation, 6-7 drops of Barrit's Reagent A were added and vortexed for 3 times for 1 minute.
- 6-7 drops of Barrit's Reagent B were added to the surface of the broth and gently the tube was shaken.
- After few minutes the color change was observed immediately.

TABLE-4: Media composition of MR-VP Broth

REAGENT	STANDARD WEIGHT (S.W) FOR 1000 ml	CALCULATED WEIGHT (C.W) FOR 20 ml
Peptone	7.0 gm/L	0.14 gm
Glucose	5.0 gm/L	0.1 gm
Dipotassium Phosphate	5.0 gm/L	0.1 gm
Distilled water	1000 ml	20 ml

4. CITRATE TEST

Procedure:

- 2 test tubes were taken and kept in hot air oven for some time to get it dried.
- By weighing the citrate agar and mixed with 20 ml distilled water, the media was prepared.
- Test tubes were cotton plugged and wrapped with newspaper and kept in a beaker with proper labelling.
- It was autoclaved and then was kept in biosafety cabinet to cool down at room temperature. Then Simmons citrate agar was poured in test tubes and kept in a slanted position.
- The slant agar was inoculated with a pure bacterial colony using a sterile loop.
- It was incubated at 35–37°C for 24–48 hours.
- The slants were observed for growth and color change.

TABLE-5: Media composition for Citrate Test

REAGENT	STANDARD WEIGHT (S.W) FOR 1000 ml	CALCULATED WEIGHT (C.W) FOR 20 ml
Simmons Citrate Agar	24.28 gm	0.48 gm
Distilled Water	1000 ml	20 ml

➢ STARCH HYDROLYSIS TEST

Principle:

Starch is a complex polysaccharide made up of amylose and amylopectin. Some bacteria secrete amylase, an extracellular enzyme that breaks down starch into smaller sugars.

After bacterial growth on starch agar, the plate was flooded with iodine, which reacts with unhydrolyzed starch to produce a blue-black or purple color.

- A clear halo around the bacterial growth indicates starch hydrolysis (positive result).
- A blue-black color around growth indicates no hydrolysis (negative result).

Procedure:

- 25 ml starch media solution was made for starch hydrolysis test by weighing the media compositions and mixed in 25 ml distilled water, the solution was prepared in a conical flask.
- The prepared solution was cotton plugged and also the test-tubes were wrapped with paper and sterilized for autoclave.
- Samples were autoclaved.
- After this step, the media was kept to cool down a bit in biosafety cabinet with all precautions.
- We poured the media in the petri dishes and left it to solidify at room temperature.
- Bacterial colony was inoculated in the solidify media through streak plate technique.
- Incubated at 35–37°C for 24 hours.

- After incubation, the plate was flooded with Gram's iodine solution.
- Color change and clear zones was observed around bacterial growth.

TABLE-6: Media composition for Starch Hydrolysis

REAGENT	**STANDARD WEIGHT (S.W)** **FOR 1000 ml**	**CALCULATED WEIGHT (C.W)** **FOR 25 ml**
Beef Extract	3 gm/L	0.075 gm
Starch	10 gm/L	0.25 gm
Agar	12 gm/L	0.3 gm

➢ UREASE TEST

Principle:

Urease breaks down urea into ammonia and CO_2.

The released ammonia increases the alkalinity of the medium, which is detected by a pH indicator (phenol red).

- Phenol red turns pink/fuchsia in alkaline pH (>8.2)
- It remains orange/yellow at acidic/neutral pH

Procedure:

- 20 ml of solution in a conical flask with Urease media composition was prepared.
- 2 test tubes were taken and kept in hot air oven for some time to get it dried.

- By weighing different components, the solution was prepared.
- Test tubes were cotton plugged and wrapped with newspaper and kept in a beaker with proper labelling.
- Test tubes are autoclaved.
- After Autoclave it was kept in biosafety cabinet to cool down at room temperature. Then the media was in test tubes and kept in the slanted positions.
- The slant surface of urea agar was streaked with a loopful of the pure bacterial culture.
- Inoculated test tube along with the uninoculated control were incubated at 35–37°C for 24 hours.
- Observed and Examined the slant for growth and color change from yellow to orange.

TABLE-7: Media composition for Urease Test

REAGENT	**STANDARD WEIGHT (S.W) for 1000 ml**	**CALCULATED WEIGHT (C.W) FOR 20 ml**
Urease	20.0 gm/L	0.4 gm
Sodium Chloride	5.0 gm/L	0.1 gm
Monopotassium Phosphate	2.0 gm/L	0.04 gm
Peptone	1.0 gm/L	0.02 gm
Dextrose/Glucose	1.0 gm/L	0.02 gm
Agar	15.0 gm	0.3 gm
Phenol Red	0.012 gm	0.012 gm

➢ GELATINASE TEST

Principle:

Gelatin is a solidifying agent and a protein. Some bacteria produce gelatinase, an

extracellular proteolytic enzyme that breaks down gelatin into smaller peptides and amino acids.

When an organism produces gelatinase, it liquefies the solid gelatin medium.

- Liquefaction of medium is Positive
- Solid medium is Negative

  Liquefaction occurs even when the tube is chilled — to confirm positive results, the tube should be refrigerated or placed on ice.

Procedure:

- 20 ml media solution was made for gelatinase test.
- By weighing the media components and mixed with 20 ml of the distilled water, the solution was prepared in a conical flask.
- The test-tubes were cotton plugged and wrapped with paper and sent it for autoclave.
- Samples were taken for autoclave at 121°C for 30 mins.
- After this step, the media was kept to cool down a bit in biosafety cabinet with all precautions.
- And then media was poured into the test tube
- The media was inoculated with the strain through inoculating loop.
- Incubated at 35–37°C for 24 hours.
- After incubation, it was refrigerated (4˚-20°C) for 20–30 minutes.
- Checked for liquefaction and observed the results.
- The tubes were tilted to check if the medium remains liquid or solid.

TABLE –8: Media composition of Gelatinase Test

REAGENT	STANDARD WEIGHT (S.W) FOR 1000 ml	CALCULATED WEIGHT (C.W) FOR 20 ml
Gelatin	120 gm/L	2.4 gm
Peptone	5 gm/L	0.1 gm
Beef extract	3 gm/L	0.06 gm

➢ CATALASE TEST

Principle:

Catalase is an enzyme produced by aerobic and facultative anaerobic bacteria to detoxify hydrogen peroxide, a harmful byproduct of aerobic metabolism.

When hydrogen peroxide is added to a bacterial culture:

- Bubbles of oxygen gas forms if catalase is present.
- No bubbles indicate a catalase-negative organism.

Procedure:

- A clean, dry glass slide was placed on a flat surface.
- Using a sterile loop, a small amount of a fresh bacterial colony was picked from the master plate and was placed on the slide in a small spot making a dry smear.
- 1–2 drops of 3% hydrogen peroxide was added directly onto the bacterial smear.
- Observed immediately for the formation of bubbles or effervescence.

➢ OXIDASE TEST

Principle:

Cytochrome c oxidase catalyzes the transfer of electrons from cytochrome c to oxygen (the final electron acceptor in aerobic respiration).

In the oxidase test:

- A chromogenic reducing agent (such as tetramethyl-p-phenylenediamine dihydrochloride) acts as an artificial electron donor.
- If cytochrome c oxidase is present, the reagent is oxidized, producing a dark purple/blue color.

Procedure:

- A piece of filter paper was moistened with a drop of oxidase reagent i.e. Gordon-Macleod reagent.
- Using a sterile inoculating loop, a small amount of the bacterial colony was picked and was smeared onto the treated area of the paper.
- Color change was observed.

➢ SUGAR FERMENTATION TEST

Principle:

Many bacteria can ferment sugars like glucose, lactose, sorbitol, fructose and maltose to produce acid and sometimes gas. The production of acid changes the pH of the medium, which is indicated by a pH indicator (usually phenol red).

- Phenol red is red/orange at neutral pH (~7.0), turns yellow in acidic pH (<6.8), and may turn pink in alkaline pH (>7.4).

Procedure:

For sugar test we have taken five sugar samples:

- Glucose
- Lactose
- Sorbitol
- Sucrose
- Maltose

  - 100 ml of media solution in a conical flask for sugar fermentation test.
  - Media for five different carbohydrates was transferred to test tubes each having one test sample and one control.
  - 10 test tubes (each containing 10 ml) were taken and cotton plugged and wrapped with paper or aluminium foil to set in autoclave in a beaker.
  - Test tubes are autoclaved at 121°C for 30 minutes.
  - After Autoclave test tubes were undergone airflow in biosafety cabinet
  - Inoculated the Medium: the 5 test tubes for different carbohydrates labelled as test sample were taken and aseptically inoculated with a loopful of the test organism.
  - Test tubes were incubated at 35–37°C for 24 hours.
  - Result was observed for any colour change.

TABLE-9: Media composition for Sugar Test

REAGENT	**STANDARD WEIGHT (S.W)** **FOR 1000 ml**	**CALCULATED WEIGHT (C.W)** **FOR 100 ml**
Peptone	10.0 gm/L	1 gm
Sodium chloride	5.0 gm/L	0.5 gm
Beef extract	1.0 gm/L	0.1 gm
Phenol Red (pH Indicator)	0.018 gm	0.018 gm

Specific carbohydrate (e.g.- glucose, lactose)	10 gm/L (1% of Sugar)	0.1 gm
Distilled water	1000 ml	100 ml (10 ml each test tube)
5M NaOH (pH- after preparation)	1-2 drops	1-2 drops

For additional confirmation, the isolate was streaked onto Mannitol Salt Agar (MSA), which favours only the growth of *Staphylococcus* species due to its high salt concentration.

➢ MSA (MANNITOL SALT AGAR)

Principle:

Mannitol Salt Agar (MSA) is both a selective and differential medium used mainly for the isolation and identification of *Staphylococcus* species, particularly *Staphylococcus aureus*.

- Selective action: Due to high salt concentration (7.5% NaCl), MSA inhibits the growth of most Gram-negative and some Gram-positive bacteria, allowing only halotolerant organisms like *Staphylococcus* spp. To grow.
- Differential action: The medium contains mannitol (a fermentable carbohydrate) and phenol red (a pH indicator). Organisms that can ferment mannitol produce acid, lowering the pH and turning the phenol red indicator yellow.

Procedure:

1. 25 ml of solution was prepared for MSA in a conical flask.
2. After the solution was prepared, wrapped with newspaper or aluminium foil to set in autoclave in a beaker.
3. The media was autoclaved.
4. After autoclaving the media was poured in the petri plate under aseptic conditions.
5. Using a sterile loop, bacterial colony was streaked in the MSA Plate.

6. Closed the lid, inverted the plate, and incubated at 37°C for 24–48 hours.
7. Growth and color change was observed in the medium.

TABLE –10: Media composition for MSA

REAGENT	**STANDARD WEIGHT (S.W) for 1000 ml**	**CALCULATED WEIGHT FOR 25 ml**
Mannitol Salt Agar	111.02 gm/L	2.77 gm
Distilled Water	1000 ml	25 ml

4. **Confirmatory decolorization**:

To confirm the decolorization potential of the obtained bacterial strain, a comparative experiment was done, where optical density (OD) of Minimal Salt Medium (MSM) broth containing only carbon source supplemented with the Methyl Red, inoculated with the selected bacterial strain was measured at different time intervals using UV-Visible spectrophotometer.

Procedure:

Medium preparation:

- The standard components of Minimal Salt Medium were mixed with 50 ml of distilled water.
- 50 ml of MSM broth was divided equally into two conical flasks, one was labelled as test sample where bacterial stain would be inoculated and other 25 ml was used as a blank during OD measurement.

Carbon source and dye solution:

- 0.25 gm of carbon source glucose was measured for each 25 ml MSM broth and was mixed after autoclave.
- 0.04 mg/ml Methyl Red dye solution was prepared for 25 ml of MSM broth labelled as test sample.

Sterilization:

- Prepared MSM broth was autoclaved.
- After autoclave, the broth was allowed to cooled down at room temperature. The carbon source was mixed with each MSM broth. The dye solution was mixed only with the MSM broth labelled as test sample.

Inoculation:

- Bacterial strain was inoculated only in the conical flask labelled as test sample.
- Another conical flask was left uninoculated to serve as a blank.

Incubation:

- The test sample was incubated at 35-37 ˚C for 24 hours.

Absorbance Measurement:

- First the uninoculated MSM broth was set as blank.
- Then one initial OD (optical density) of test sample was measured at 0 hour that is just after inoculation.
- Then OD of the test sample was measured at regular intervals (e.g. 30 mins, 60 mins, 90 mins).

Calculation of decolorization efficiency:

Percentage decolorization was calculated using the following formula:

$$\text{Degradation } (\%) = \frac{(\text{Initial OD} - \text{Final OD})}{\text{Initial OD}} \times \times 100$$

Where initial OD is the absorbance of sample at 0 hour (before degradation)

And Final OD is the absorbance of the sample after degradation

A greater decolorization efficiency indicates a better ability of the organism or the condition tested to degrade or remove the dye from the solution. This measure is important for implementing bioremediation agents for environmental scale.

5. **Optimization**:

To assess the influence of environmental factors on the efficiency of dye decolorization by the bacterial strain, three key factors including pH, temperature and salinity were optimized individually. In each case, Minimal Salt Medium (MSM) containing carbon source and Methyl Red dye solution was used for screening and absorbance was measured at specific intervals using UV-Visible spectrophotometry.

A. Optimization of pH:

Procedure:

Media preparation:

- 50 ml of distilled water was mixed with the components of MSM broth.
- The broth was equally (25 ml each) divided into two conical flasks.
- Before autoclaving and adding dye solution, the pH of the solution was measured.
- The pH of the broth was adjusted to two different pH levels:
  - 3-4 drops of 1 N HCl were added into one conical flask and pH was adjusted to pH 5 (acidic).
  - 3-4 drops of 1 N NaOH were added into another conical flask and the pH was adjusted to pH 11 (basic).

Sterilization:

- Prepared MSM broth was sterilized using autoclave at 121 ˚C for 15 minutes.
- After autoclave, the broth was cooled down to room temperature and the carbon source and dye solution was mixed with the MSM broth of each conical flask.

Inoculation:

- The bacterial strain was inoculated into each flask under aseptic conditions.

Incubation:

- Flasks were incubated at 35-37 ˚C for different time periods (e.g. 1hr, 2hr, 3hr).

Monitoring:

- At regular intervals (e.g. 0,1,2,3,4 hours) samples were withdrawn and absorbance was measured at 428.87nm using a UV-Visible spectrophotometer.

Calculation of decolorization efficiency:

Percentage decolorization was calculated using the given formula.

B. Optimization of Temperature:

Procedure:

Media preparation:

- 50 ml of distilled water is mixed with the components of MSM broth.
- The broth was equally (25 ml each) divided into two conical flasks.
- The pH was adjusted to neutral.

Sterilization:

- Prepared MSM broth was sterilized using autoclave at 121 ˚C for 15 minutes.
- After autoclave, the broth was cooled down to room temperature and the carbon source and dye solution was mixed with the MSM broth of each conical flask.

Inoculation:

- The bacterial strain was inoculated into each flask under aseptic conditions.

Temperature conditions:

- Flasks were incubated at two different temperatures: 30 ˚C and 60 ˚C for the same time periods.

Monitoring:

- Samples were taken at specific time intervals (e.g. 0, 1, 2, 3 hours) and absorbance was measured at 428.87nm using UV-Visible spectrophotometer to evaluate decolorization efficiency of both the sets.

Calculation of decolorization efficiency:

Percentage decolorization was calculated using the given formula.

C. Optimization of Salinity:

Procedure:

Media preparation:

- Four conical flasks were taken, each containing 50ml of distilled water mixed with every component of MSM but each broth containing four different concentrations of sodium chloride (NaCl):
  - One flask contained no salt.
  - One flask contained 0.005 gm of salt
  - One flask contained 0.01 gm of salt
  - One flask contained 0.1 gm of salt

Sterilization:

- Prepared MSM broth was sterilized using autoclave at 121 ˚C for 15 minutes.
- After autoclave, the broth was cooled down to room temperature and the carbon source and dye solution was mixed with the MSM broth of each conical flask.

Inoculation:

- The bacterial strain was inoculated into each flask under aseptic conditions.

Incubation:

- Flasks were incubated at 35-37 ˚C for different time periods (e.g. 1hr, 2hr, 3hr).

Monitoring:

- At regular intervals (e.g. 0,1,2,3,4 hours) samples were withdrawn and absorbance was measured at 428.87nm using a UV-Visible spectrophotometer.

Calculation of decolorization efficiency:
Percentage decolorization was calculated using the given formula.

D. Selection of optimal conditions for safety analysis:

These optimal parameters were therefore selected and used as standard conditions for

conducting the subsequent safety analysis experiments to assess the behavior and potential risk associated with the textile dye Methyl Red under effective decolorization conditions.

6. Safety Analysis:

To assess the environmental safety of dye and its treated forms under optimized decolorization conditions, a seed germination bioassay was conducted using green Mung seeds (*Vigna radiata*).

Procedure:

- Five sterile petri plates were prepared and labelled according to treatment conditions. In each plate, pre-washed green mung seeds were placed on clean tissue papers. Each plate received one of the following treatment solutions:

    1. Water: Seeds were treated with only tap water.
    2. Dye solution: Seeds were treated with untreated dye solution.
    3. Dye solution treated at 60 ˚C temperature: Seeds were treated with dye solution inoculated with bacterial strain, after 24 hours incubation at 60˚C.
    4. Basic dye solution: Seeds were treated with dye solution adjusted to pH 11 after 24 hours of incubation with bacterial strain.
    5. Salinity treated dye: Seeds were treated with dye solution supplemented with 0.1 gm in 50 ml of NaCl.

- The seeds were treated with those solutions continuously for seven days to observe the germination process.
- After seven days the length of the roots germinated from the seeds were measured.

RESULTS AND DISCUSSION

1. Isolation of the bacterial strain: -

- Serial dilution:

  - Result:

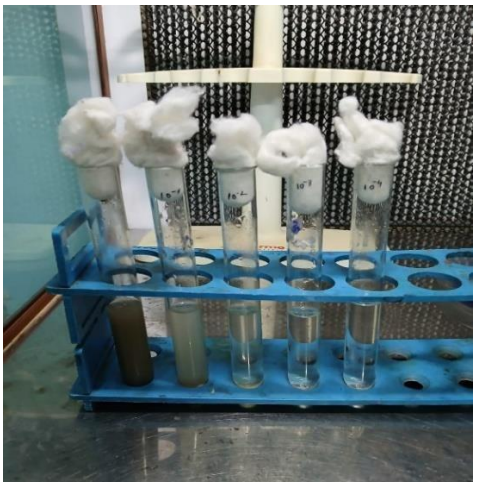


Fig 4: Serial dilution of the collected soil

Discussion:

The serial dilution of the original sample was performed up to the dilution factor of 10^{-4}. The turbidity of the tubes decreased progressively with increasing dilution, as follows:

- Tube 10^{-1}: highly turbid, indicating dense, microbial growth.
- Tube 10^{-2}: moderately turbid.
- Tube 10^{-3}: slight turbidity observed.
- Tube 10^{-4}: almost clear, indicating minimal microbial presence.

This gradation of turbidity confirms the expected pattern of dilution: as the concentration of the microbial culture decreases, so does the optical density.

- Spreading on agar plate: -

  Result:

Fig 5: Spreading on nutrient agar treated with Methyl Red

Discussion:

The plate displayed numerous well isolated bacterial colonies after 24 hours of incubation. It was observed that some clear zones were there around the growth of the bacterial colony. This indicated active dye decolorization by bacterial culture. The extent of colorization was visually evident across the plate, suggesting degradation of the methyl red dye. The decolorization of methyl red dye by bacterial isolate indicated its potential in bioremediation of azo dyes. The positive result confirms the bacterial strain's capability to tolerate and metabolize synthetic azo dye methyl red.

- Streaking:

  Result:

Fig 6: Sample streaking on nutrient agar plate containing Methyl Red dye

Discussion:

Bacterial isolate from dilution of 10^{-4} was streaked onto nutrient agar supplemented with Methyl Red dye, showed visible zone of decolorization along with bacterial growth. The

decolorized regions indicated successful dye degradation. The ability of the bacterial isolate to decolorize methyl red dye was observed. The experiment results showed positive decolorization indicating the potential of the bacterial strain for dye degradation.

- Preparation of master plate:

  Result:

Fig 7: Master plate

Discussion:

A master plate was successfully prepared using streak plate technique from the bacterial colony taken from the previously streaked plate. Uniform distribution of the bacterial growth was observed. This master plate was then used as a primary source for transferring identical patterns of colonies fur further experimental tests.

2. Preliminary decolorization: -

Result:

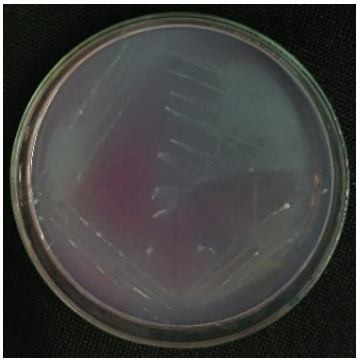


Fig 8: Streaking on MSM agar containing only carbon source

Discussion:

Clear formed zones along the streak lines indicated degradation of the methyl red dye. It was observed that the degradation capability of microbial stain was much better on the MSM media containing only carbon source than MSM containing nitrogen source. The decolorization zones are more prominent on the carbon containing MSM. This suggested that the carbon source plays a vital role in supporting bacterial growth and metabolic activity., thereby enhancing the decolorization efficiency.

This preliminary experiment indicated that the supplementation of a carbon source is important for optimal dye decolorization by selected bacterial strain and was considered in further optimization studies.

3. Identification of isolated strain: -

- **Gram staining:**

  Result:

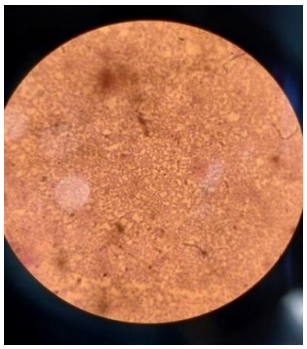


Fig 9: Gram staining

Discussion:

After performing gram staining of the bacterial isolate from the master plate, the cells were appeared as purple colored, round, cocci-shaped formations. This observation supported the preliminary identification of the bacterial isolate as a member of the genus *Staphylococcus*.

- Biochemical test: -

  IMVIC Test: -

  - **INDOLE TEST:**

Result:

Fig 10: Indole test

Discussion:

The indole test showed a red ring formation on the surface of the tube. This indicated a positive indole test. This
supported the identification of the isolate as *Staphylococcus* sp.

- **METHYL RED TEST:**

Result:

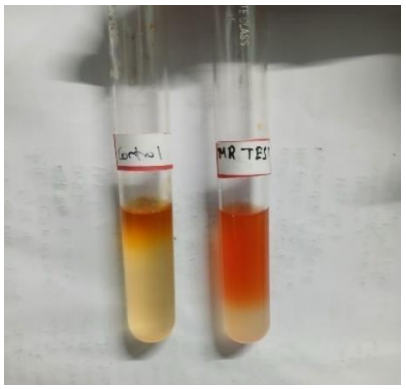


Fig 11: Methyl Red test

Discussion:

After adding Methyl Red indicator, the test tube developed a distinct red coloration, indicating a positive MR test this result suggested that the isolate performed acid fermentation producing acid to lower the pH.

- **VOGES- PROSKAUER TEST:**

Result:

Fif 12: Voges-Proskauer test

Discussion:

After addition of Barritt's reagents A and B and incubation, a reddish-pink coloration in the upper was seen indicating a positive VP test. That suggested that the bacterial strain was capable of fermenting glucose, resulting in the production of acetoin, indicating that the bacterial strain could be the *Staphylococcal* sp.

- **CITRATE TEST:**

Result:

Fif 13: Citrate test

Discussion:

the test showed a color change from green to blue, indicating a positive result. This confirms that the bacterial isolate was capable of using citrate as the sole carbon source, suggesting that the bacterial strain could be a genus of *Staphylococcus*.

- **STARCH HYDROLYSIS TEST:**

Result:

Fig 14: Starch Hydrolysis test

Discussion:

After incubation, the starch agar plate was flooded with iodine solution. A clear zone was appeared around the bacterial growth, indicating a positive result. This suggested that the isolate presumably *Staphylococcus* sp.

- **UREASE TEST:**

Result:

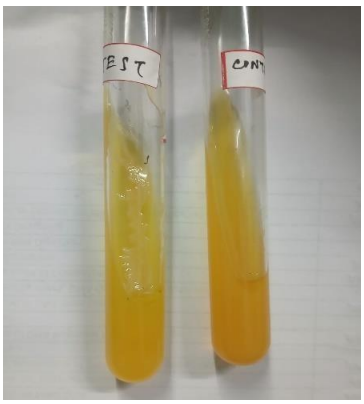


Fig 15: Urease test

Discussion:

After incubation, the color of the urea agar slant remained yellow, indicating a negative result for urease activity. This test signifies that the bacterial isolate is incapable of producing the enzyme urease. This suggested that urea is not utilized as a nitrogen source by the organism.

- **GELATINASE TEST:**

Result:

Fig 16: Gelatinase test

Discussion:

After incubation and refrigeration of the gelatin stab tubes, the medium remained solid, indicating a negative test result. There was no liquefaction of the gelatin. A negative test indicated that the bacterial isolate did not produce the enzyme gelatinase.

- **CATALASE TEST:**

Result:

Fig 17: Catalase test

Discussion:

When hydrogen peroxide was added to the culture of the bacterial isolate, immediate bubbling was observed due to rapid release of oxygen gas, indicating positive catalase test. It confirmed that bacterial isolate could produce catalase enzyme, suggesting that the bacterial strain was presumably *Staphylococcus* sp.

- **OXIDASE TEST:**

Result:

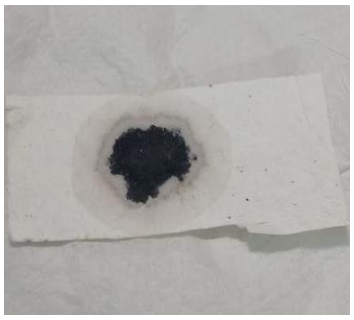


Fig 18: Oxidase test

Discussion:

When bacterial isolate was tested using oxidase reagent, no color change to dark purple was observed, indicating a negative oxidase test. This result was consistent with the general biochemical profile of *Staphylococcus* sp

- **SUGAR FERMENTATION TEST:**

Result:

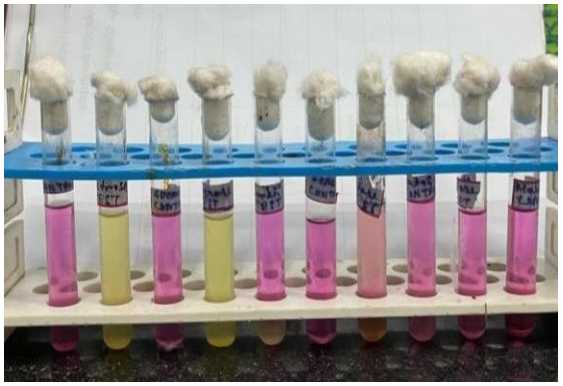


Fig 19a: Sugar fermentation test

Fig 19b: Dextrose test

Fig 19c: Lactose test

Fig 19d: Sucrose test

Fig 19e: Sorbitol test

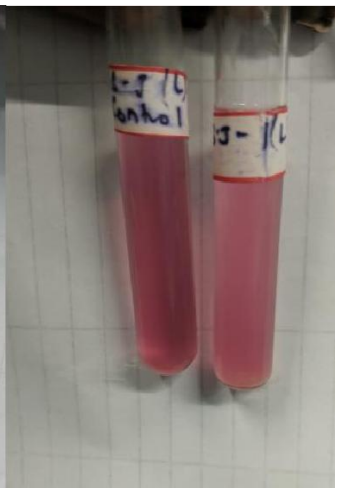


Fig 19f: Maltose test

Discussion:

The bacterial isolate was tested for sugar fermentation test, where dextrose and sucrose showed color change from red to yellow, indicating acid production thus, a positive fermentation result. Whereas lactose, sorbitol and maltose showed no color change indicating no acid fermentation and

therefore, negative fermentation result.

Based on the results of all biochemical tests performed, the bacterial isolate was presumptively identified as a member of *Staphylococcus* genus. For additional confirmation, the isolate was streaked onto Mannitol Salt Agar (MSA), which favors only the growth of *Staphylococcus* species due to its high salt concentration

- **MSA TEST:**

Result:

Fig 20: MSA Tes

Discussion:

The bacterial isolate grew well on Mannitol Salt Agar indicating a positive result. That suggested that the organism can grow in the presence of 7.5% NaCl, which is selective for *Staphylococcus* sp. This result supported the presumptive identification of the isolate as a *Staphylococcus* species, particularly *Staphylococcus aureus*.

Growth on Mannitol Salt Agar with a yellow coloration of the medium confirmed the isolate as *Staphylococcus aureus* as indicated by the mannitol fermentation characteristic of this species.

4. Confirmatory decolorization:

Result:

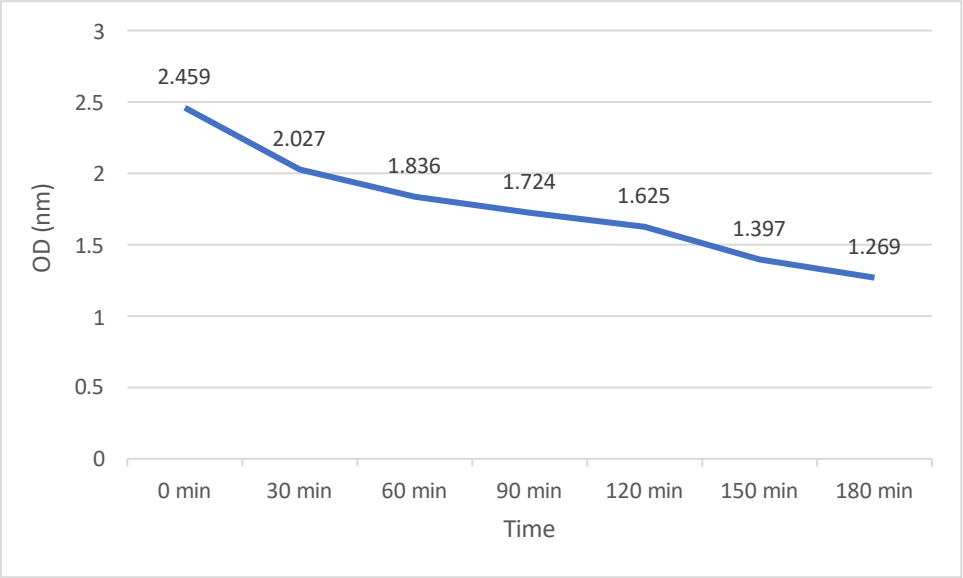


Fig 21: Absorbance of MSM broth taken at 428.87nm at different time intervals

the decolorization potential of the *Staphylococcus aureus* strain was assessed using Minimal Salt Medium (MSM) supplemented with carbon source glucose and methyl red azo dye. The optical density (OD) was measured at 428.87 nm at different time intervals to monitor the degradation of methyl red dye.

Discussion:

The data indicated a gradual decline of OD values over time, which implied successful decolorization of methyl red dye by the bacterial strain. The OD dropped from 2.459 at 0 minutes to 1.269 at 180 minutes, which proved that the strain can degrade the dye under the experimental conditions. The fastest decolorization occurred during the first 30 minutes which indicated that the bacterial strain rapidly degraded the dye during the initial period, thereafter continued to degrade Methyl Red at a slower but sustained degradation rate until the end of the 180 minutes.

Degradation (%) = (Initial OP-Final OD)/(Initial OD) ×100

= [(2.459 – 1.269)/2.459]*100

= 48 %

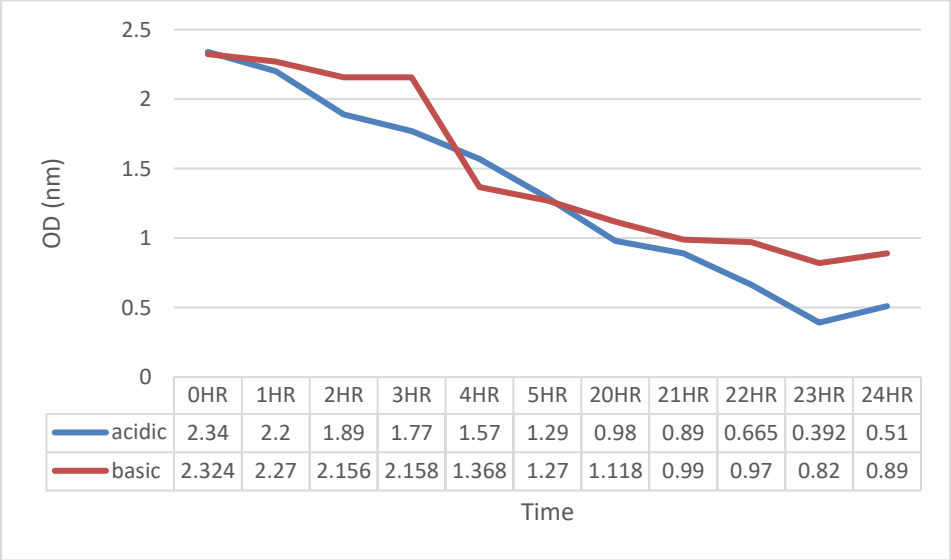
This result from the experiment confirmed that the bacterial strain was capable of enzymatic activity that cleave azo bonds. The use of MSM also showed that the strain can efficiently degrade methyl ed even in low nutrient conditions which suggested that it was well suited for bioremediation applications.

5. Optimization: -

- Optimization Of pH:

  Result:

The decolorization efficiency of the *Staphylococcus aureus* strain under acidic (pH 5) and basic (pH 11) conditions was monitored over a 24 hours period by measuring optical density (OD) at 428.87 nm. The OD values for both conditions showed a general decreasing trend, indicating successful dye decolorization over time.



	0HR	1HR	2HR	3HR	4HR	5HR	20HR	21HR	22HR	23HR	24HR
acidic	2.34	2.2	1.89	1.77	1.57	1.29	0.98	0.89	0.665	0.392	0.51
basic	2.324	2.27	2.156	2.158	1.368	1.27	1.118	0.99	0.97	0.82	0.89

Fig 22: pH effect on the degradation efficiency of methyl red dye

Acidic condition (pH 5) began with an OD of 2.34 at 0 hours and steadily declined to 0.51 at 24 hours.

Basic condition (pH 11) started at 2.324 and decreased to 0.89 at 24 hours.

The decrease in OD under both the pH conditions confirmed the decolorization activity.

Although, the acidic condition showed a more consistent and prominent reduction in OD values compared to the basic condition.

Discussion:

The result indicated that pH significantly affected the dye degrading efficiency of the *Staphylococcus aureus* strain. the acidic environment facilitated a more uniform and pronounced decolorization, as evidenced by the steady decline in OD values across all the time intervals. This suggested that the enzymatic activity or the metabolic activities of the *Staphylococcus aureus* strain, responsible for dye breakdown might be more active or stable at lower pH levels. In contrast, the basic condition also supported degradation of the Methyl Red dye, but it was less consistent with minor fluctuations.

Decolorization efficiency of acidic pH:

Degradation (%) = (Initial OP-Final OD)/(Initial OD) ×100

= [(2.34 – 0.51)/2.34]*100

= 78%

Decolorization efficiency of basic pH:

Degradation (%) = (Initial OP-Final OD)/(Initial OD) ×100

=[(2.324 – 0.89)/2.324]*100

= 61%

Both conditions showed effectiveness in decolorization but the acidic condition was more reliable for consistent performance.

- Optimization of temperature:

  Result:

The effect of temperature on methyl red dye degradation by the Staphylococcus aureus strain was studied by comparing the OD at 428.87 nm over a 4 hours time interval under two different temperature conditions.

Fig 23: Temperature effect on the degradation efficiency of methyl red dye

At 60˚C, the OD reduced from 2.21 at 0 hour to 1.02 at 4 hours.

At 30˚C, the OD decreased from 2.21 to 1.54 at 4 hours.

Both conditions demonstrated a decrease in OD, indicating degradation of methyl red dye. However, the reduction was more prominent at 60˚C, suggesting higher decolorization efficiency at higher temperature.

Discussion:

Temperature is a critical factor influencing microbial metabolism and enzyme activity during biodegradation process.

Decolorization efficiency at 60℃:

Degradation (%) = (Initial OP-Final OD)/(Initial OD) ×100

= [(2.21 – 1.02)/2.21]*100

= 54%

Decolorization efficiency at 30℃:

Degradaion (%) = (Initial OP-Final OD)/(Initial OD) ×100

= [(2.21 – 1.54)/2.21]*100

= 30%

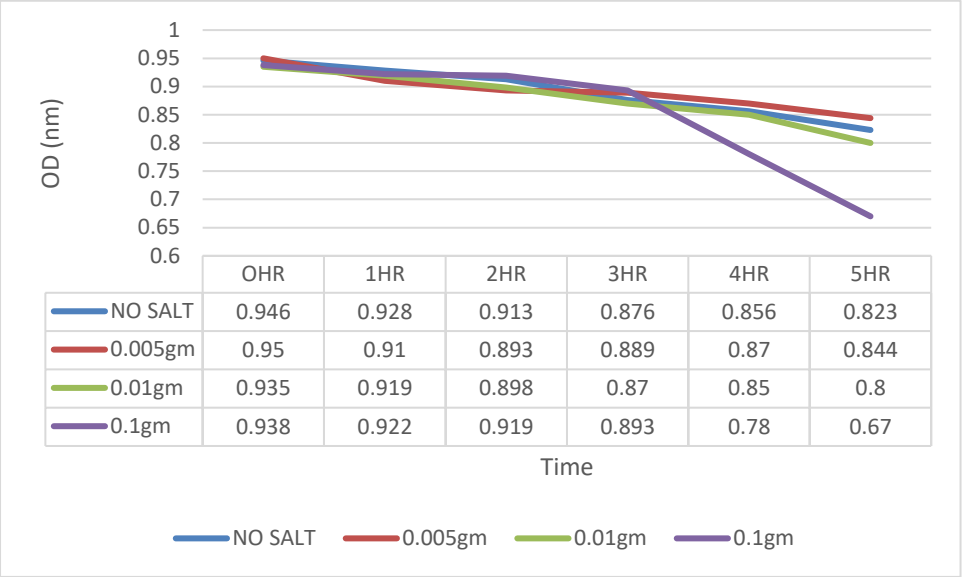
From the above result, it was clearly indicated that 60˚C is more favorable temperature for efficient dye degradation.

The consistent decline in OD values at 60˚C suggested that the dye breakdown was more active at higher temperature.

In contrast, 30˚C showed slower decolorization. While decolorization occurred under both conditions, higher temperature significantly enhanced the rate and extent of dye removal.

- Optimization of salinity:

  Result:



	OHR	1HR	2HR	3HR	4HR	5HR
NO SALT	0.946	0.928	0.913	0.876	0.856	0.823
0.005gm	0.95	0.91	0.893	0.889	0.87	0.844
0.01gm	0.935	0.919	0.898	0.87	0.85	0.8
0.1gm	0.938	0.922	0.919	0.893	0.78	0.67

Fig 24: Salinity effect on the degradation efficiency of methyl red dye

To evaluate the effect of salinity on dye decolorization efficiency, MSM with dye was supplemented with different NaCl concentrations: no salt, 0.005 g, 0.01 g, and 0.1 g per 50 mL medium. The OD values were recorded at 428.87 nm at different time intervals for 5 hours. The results are plotted in the figure above.

No salt: optical density went from 0.946nm to 0.823nm

0.005 g: optical density went from 0.950nm to 0.844nm

0.01 g: optical density went from 0.935nm to 0.800nm

The most significant decrease in OD was observed in the sample with 0.1 g NaCl, where the OD dropped from 0.938 (0hr) to 0.67 (5hr), indicating the highest level of decolorization.

Discussion:

The result showed that salinity had a notable impact on the efficiency of dye decolorization. Among the tested concentrations, 0.1 g NaCl facilitated the highest rate of OD reduction, indicating improved degradation. This suggested that the *Staphylococcus aureus* strain used in this study, performed optimally in a moderately saline environment, likely due to enhanced

enzyme stability or osmotic balance at high salt concentration.

Decolorization efficiency with no salt:

Degradation $(\%) = \frac{Initial\ OP - Final\ OD}{Initial\ OD} \times 100$

= [(0.946 – 0.823)/0.946]*100

= 13%

Decolorization efficiency with 0.005gm NaCl:

Degradation $(\%) = \frac{Initial\ OP - Final\ OD}{Initial\ OD} \times 100$

= [(0.95 – 0.844)/0.95]*100

= 11%

Decolorization efficiency with 0.01gm NaCl:

Degradation $(\%) = \frac{Initial\ OP - Final\ OD}{Initial\ OD} \times 100$

= [(0.935 – 0.8)/0.935]*100

= 14%

Decolorization efficiency with 0.1gm NaCl:

Degradation $(\%) = \frac{Initial\ OP - Final\ OD}{Initial\ OD} \times 100$

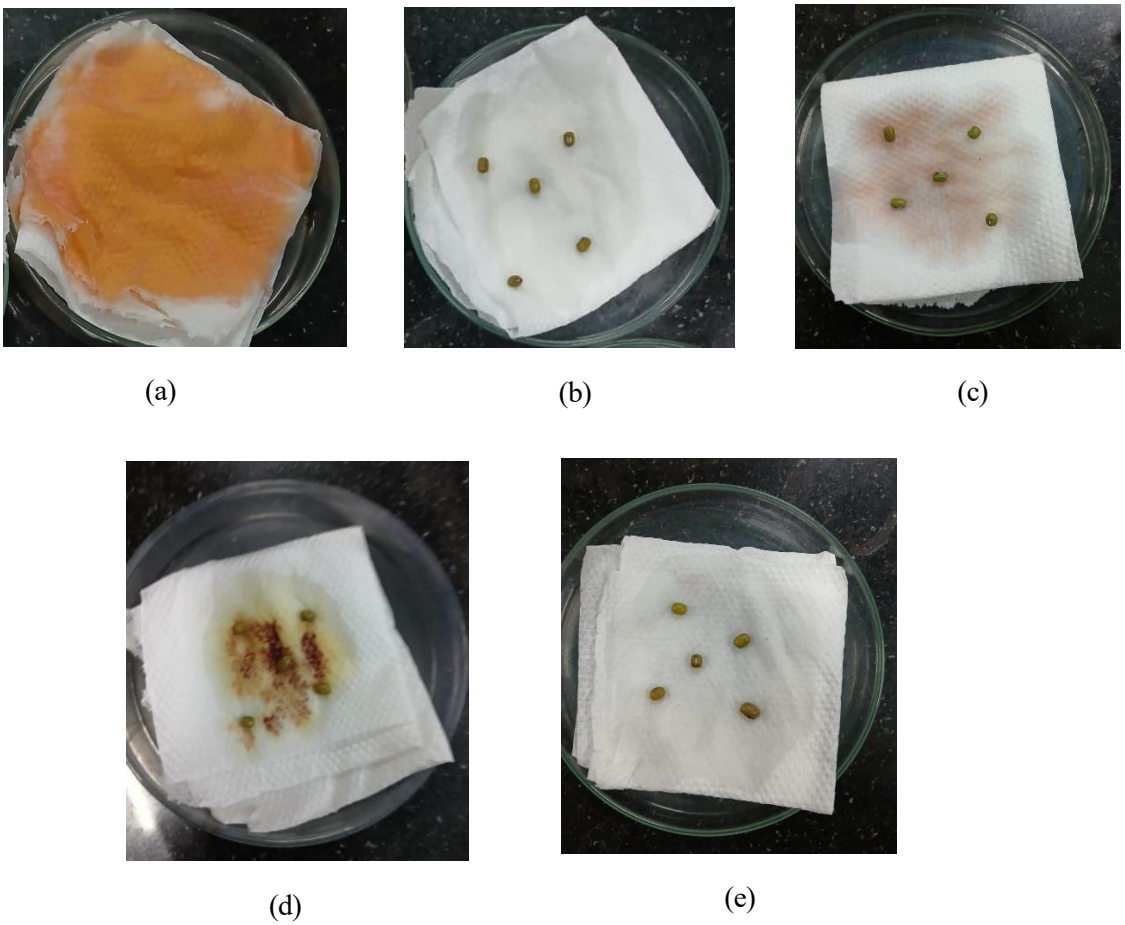
= [(0.938 – 0.67)/0.938]*100

= 28%

Based on these observations, 0.1 g NaCl was selected as the optimized condition for further experiments, including the safety analysis, as it provided the most efficient dye removal performance.

6. Safety analysis: -

Result:



(a) (b) (c)

(d) (e)

Fig 25: Seeds treated with (a) dye solution at 60˚C, (b) dye solution with 0.1gm salinity, (c) basic dye solution, (d) untreated dye solution, (e) water

Following conditions were found to yield the highest decolorization efficiency by the *Staphylococcus* strain:

- pH 11
- Temperature: 60 ˚C
- 0.1 gm of NaCl in 50 ml MSM

A seed germination assay was conducted to assess the toxicity of dye-treated solutions under various optimized and non-optimized conditions. Five treatments were tested:

- Water,

- Untreated Methyl Red dye solution,
- Dye solution treated at 60˚C,
- Dye solution treated with 0.1 gm NaCl
- Basic pH-treated dye solution.

(a) (b) (c)

(d) (e)

Fig 26: Seeds germinated after 7 days (a) dye solution treated at 60˚C, (b) dye solution with 0.1 gm salinity. (c) basic dye solution, (d) untreated dye solution, (e) water

After 7 days, the root length of the germinated seeds was measured to assess growth performance. The root length was highest at the seeds treated with simple water, then

seeds treated with 60˚C-treated dye solution, then at salt-treated dye solution. The lowest root length was seen with the seeds treated with untreated dye solution. This confirmed that the untreated dye retained highest level of toxicity, reducing germination and root growth.

Discussion:

The seed germination assay provided a simple and effective bioassay for evaluating the toxicity of dye and its treated forms. Water showed normal germination and longest root growth, establishing a reliable baseline. In contrast, the untreated dye sample showed poor germination, visible seed damage, and the shortest root length, confirming its strong toxicity.

The 0.1 g NaCl-treated dye solution demonstrated the best detoxification performance in terms of germination rate, though the root length was slightly shorter than in the 60°C- treated group. This suggested that while salinity optimization effectively reduces toxicity for initial germination, temperature treatment may promote slightly better root elongation, possibly by breaking dormancy.

The basic pH-treated dye showed partial detoxification, evident in moderate germination and short root length, while the 60°C treatment showed a most effective result, despite visible dye residue, the root growth was better than in salt or pH treatments, possibly due to reduced viscosity or dye degradation into less inhibitory by-products.

CONCLUSION

The current study evaluated the decolorization and detoxification ability of *Staphylococcus aureus* on the synthetic dye Methyl Red under varying physicochemical factors—temperature, pH, and salinity—as well as the feasibility of utilizing the workforce treatment methods by evaluating the treated dye solutions for safety using the seed germination bioassay of green mung seeds (*Vigna radiata*).

The spectrophotometric analysis illustrated that methyl red degradation efficacy was significantly enhanced by elevated temperature (60°C), basic pH (pH 11), and moderate salinity (0.1 g NaCl). However, of the three conditions, salinity demonstrated the most consistent decrease in optical density, suggesting that *Staphylococcus aureus* was able to breakdown methyl red effectively. The biological toxicity determined through the seed germination and root growth assay confirmed the untreated dye was highly toxic; however, the optimized treatments—specifically the thermal and the saline—generated reduced toxicity as per the observation of increased seed germination and root length.

In conclusion, *Staphylococcus aureus* demonstrates good potential as a biological agent to treat dye effluents, especially with straightforward optimization techniques. This biological treatment is environmentally friendly, low-cost, and practicable for small-scale remediation.

In the future, this study could be replicated on a larger scale for industrial remediation, investigate the enzymatic or genetic basis for dye degradation in *Staphylococcus aureus*, or change synthetic dye types. Ecotoxicological studies are important in other plants or aquatic model organisms to check whether the treated effluent is environmentally safe. These evaluations would aid in developing waste management systems with environmental and efficiency standards for textile and dye industries.

APPENDIX

TOPIC	PAGE NO.
Azo dye	11, 12, 13, 15, 38, 47, 59
Serial dilution	5, 37
Master plate	5, 17, 18. 20, 22, 23, 28, 38, 39, 40
MSM	5, 6, 7, 18, 19, 32, 34, 35, 36, 39, 47, 52, 54
Sugar fermentation	5, 29, 44, 45
MSA	5, 6, 7, 30, 31, 46,
Seed germination	1, 2, 3, 8, 9, 12, 36, 53, 55, 56
Gram staining	5, 19, 39
Staphylococcus aureus	1, 2, 3, 46, 47, 48, 50, 52, 56,
Dye degradation	8, 14, 16, 17, 50, 55, 56
Synthetic dyes	8, 11, 14, 15, 16
Salinity	5, 8, 9, 13, 15, 16, 34, 35, 36, 52, 53, 54, 55, 56
Catalase	5, 28, 43, 44
Toxicity	1, 2, 3, 8, 9, 11, 14, 53, 54, 55, 56
Decolorization	1, 2, 3, 12, 15, 32, 33, 34, 35, 36, 38, 39, 47, 48, 49, 50, 51, 52, 53, 53, 56, 58, 59
Optimization	1, 2, 3, 15, 16, 34, 35, 36, 39, 48, 50, 52, 55, 56, 58
Optical density	7, 8, 9, 15, 32, 47, 48, 52
Methyl Red	1, 2, 3, 5, 7, 11, 13, 15, 17, 18, 21, 22, 32, 34, 36, 37, 38, 39, 40, 41, 47, 48, 50, 52, 53, 56, 58, 59

REFERENCES

Abioye, O. P., Iroegbu, V. T., & Aransiola, S. A. (2015). Biodegradation of methyl red by *Staphylococcus aureus* isolated from waste dump site. Journal of Environmental Science and Technology, 8(3), 131–138. https://doi.org/10.3923/jest.2015.131.138

Akansha, K., Chakraborty, D., & Sachan, S. G. (2019). Decolorization and degradation of methyl orange by *Bacillus stratosphericus* SCA1007. Biocatalysis and Agricultural Biotechnology, 18, 101044. https://doi.org/10.1016/j.bcab.2019.101044

Balapure, K., Bhatt, N., & Madamwar, D. (2015). Mineralization of reactive azo dyes present in simulated textile wastewater using down flow microaerophilic fixed film bioreactor. Bioresource Technology, 175, 1-7. https://doi.org/10.1016/j.biortech.2014.10.040

Benkhaya, S., M'rabet, S., & El Harfi, A. (2020). Azo dye degradation: An overview of its mechanisms and impacts. Heliyon, 6(12), e03464. https://doi.org/10.1016/j.heliyon.2020.e03491

Chen, Y., Liu, Z., & Hu, Y. (2018). Revealing the degrading possibility of methyl red by two thermophilic bacteria. Chemosphere, 219, 920–928. https://doi.org/10.1016/j.chemosphere.2018.12.092

Chung, K. T. (2016). Azo dyes and human health: A review. Journal of Environmental Science and Health, Part C, 34(4), 233–261. https://doi.org/10.1080/10590501.2016.1236602

Garg, S. K., & Tripathi, M. (2017). Microbial strategies for discoloration and detoxification of azo dyes from textile efluents. Research Journal of Microbiology, 12(1), 1–19. http://dx.doi.org/10.3923/jm.2017.1.19

Ikram, M., Naeem, M., Zahoor, M., Rahim, A., Hanafiah, M. M., Oyekanmi, A. A., Shah, A. B., Mahnashi, M. H., Al Ali, A., Jalal, N. A., Bantun, F., & Sadiq, A. (2022). Biodegradation of azo dye methyl red by *Pseudomonas aeruginosa*: Optimization of process conditions. International Journal of Environmental Research and Public Health, 19(16), 9962. https://doi.org/10.3390/ijerph19169962

Islahuddin, N. K. S., Halmi, M. I. E., Manogaran, M., & Shukor, M. Y. (2017). Isolation and culture medium optimisation using one-factor-at-a-time and response surface methodology on the biodegradation of the azo-dye Amaranth. Bioremediation Science and Technology Research, 5(2), 25–31. https://doi.org/10.54987/bstr.v5i2.360

Malik, A., & Saha, T. (2017). Biodegradation of methyl red by alkaliphilic, halotolerant *Nesterenkonia lacusekhoensis* EMLA3: Application in alkaline and salt-rich dyeing efluent treatment. Extremophiles, 21(3), 479–490. https://doi.org/10.1007/s00792-017-0918-2

Pinheiro, H. M., Touraud, E., & Thomas, O. (2004). Aromatic amines from azo dye reduction: Status review with emphasis on direct UV spectrophotometric detection in textile industry wastewaters. Dyes and Pigments, 61(2), 121–139. https://doi.org/10.1016/j.dyepig.2003.10.009

Pirkarami, A., & Olya, M. E. (2017). Removal of dye from industrial wastewater with an emphasis on improving economic efficiency and degradation mechanism. Environmental Science and Pollution Research, 24(1), 620–637. https://doi.org/10.1016/j.jscs.2013.12.008

Rawat, D., Mishra, V., & Sharma, R. S. (2018). Detoxification of azo dyes in the context of environmental processes. Chemosphere, 211, 149-162. https://doi.org/10.1016/j.chemosphere.2016.04.068

Robinson, T., McMullan, G., Marchant, R., & Nigam, P. (2005). Removal of dyes from the effluent of textile and dyestuff manufacturing industry: A review of emerging techniques with reference to biological treatment. Critical Reviews in Environmental Science and Technology, 35(3), 219–238. https://doi.org/10.1080/10643380590917932

Sharma, S., Sun, Q., Li, J., & Yu, C.-P. (2016). Decolorization of azo dye methyl red by suspended and co-immobilized bacterial cells with mediators anthraquinone-2,6-disulfonate and Fe_3O_4 nanoparticles. Process Biochemistry, 51(5), 700–707. https://doi.org/10.1016/j.procbio.2016.01.007

Shahid, M., Khan, M. I., Khan, S. B., & Mohammad, F. (2013). Recent advancements in natural dye applications: A review. Journal of Cleaner Production, 53, 310–331. https://doi.org/10.1016/j.ibiod.2013.06.002

Singh, R. S., Sarbhoy, R. K., Singh, G., Sarabhai, A., & Kumar, P. (2013). Toxic effect of dyes on seed germination and early seedling growth of Zea mays. Indian Research Journal of Genetics and Biotechnology, 5(3), 194–196. https://irjgbt.in/index.php/IRJGBT/article/view/130

SmallSEOTools

Plagiarism Scan Report By SmallSEOTools

Report Generated on: May 27,2025

Total Words: 1000 Total Characters: 6683 Plagiarized Sentences: 5.16 Unique Sentences: 37.84 (91%)

Content Checked for Plagiarism

SHRM Bio-technologies Pvt Ltd CINNO–U40107WB2006PTC110903 MSMENo–WB14D0013023 Honorary Recognization From LSSSDC, NSDC, Govt. of India. An ISO 9001:2015 Certi ed Organisation Humaipur. PO: Abdalpur Madhyamgram Kolkata 700155 Phone: +918981003215 Email:info@shrmbio.com www.shrmbio.com 2 Ref:-SHRM/WB/1404-06/ Date:-30/06/2025 CERTIFICATE This is to certify that Mr./ Miss Paulami Chakraborty, student of Sister Nivedita University Reg. No.- 230010426122 has completed the 6 months Project work, entitled "Optimization of Methyl Red Dye Decolorization by Staphylococcus aureus and Toxicity Assessment via Seed Germination Bioassay", under the guidance of Pranati Das at SHRM Biotechnologies Pvt. Ltd., Madhyamgram, West Bengal. We wish her all the best for her future endeavors. As a student she has proved herself diligent, sincere and well behaved. Mr. Kunal Vora Pranati Das Center in Charge Project Co-ordinator SHRM Biotech SHRM Biotech Awarded the Best Biotech Training provider in Eastern India- 2018 by Union Minister of Agriculture AwardedTrainingExcellenceAwardonLifeSciencesbyHon'ble governorsofOrissa&Tripurain2017 DECLARATION BY STUDENT I hereby declare that the work which is being presented in this dissertation entitled "Optimization of Methyl Red Dye Decolorization by Staphylococcus aureus and Toxicity Assessment via Seed Germination Bioassay " in Wthe partial ful lment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from December to May under the supervision of Ms. Pranati Das, Research and training coordinator, at SHRM BIOTECHNOLOGIES PVT LTD, MADHYAMGRAM. I acknowledge that I have read and understood the UGC regulations, 2018 (Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions). I con rm that this dissertation has been checked for plagiarism using the plagiarism checking software- . At the end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential source (if any), generated online using plagiarism checking software is enclosed. I herewith con rm that the dissertation has been less than 10% similarity according to the plagiarism checking software's report and meets the UGC Regulations as well as plagiarism rules of Sister Nivedita University. I further declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and ndings described in my dissertations. (Signature of the students with Date) PAULAMI CHAKRABORTY REGISTRATION NO 230010426122 MSc. MICROBIOLOGY (2023-2025) 2 TO WHOM IT MAY CONCERN This is to certify that the project report titled " Optimization of Methyl Red Dye Decolorization by Staphylococcus aureus and Toxicity Assessment via Seed Germination Bioassay ", submitted by Mr./Ms. Paulami Chakraborty (Registration No. 230010426122 ,Enrolment No. 2322211001042) to the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona de record of research conducted by him/her for the partial ful lment of the requirements for the Master of Science degree.

Date: Place: Kolkata _____ Signature of Head of the Department Seal ACKNOWLEDGMENT I, Paulami Chakraborty would like to express my sincere gratitude to all those who supported and guided me throughout the completion of this project. First and foremost, I would like to thank Ms. Pranati Das, Research and Training Co-Ordinator, SHRM Biotechnologies Pvt Ltd, for their invaluable guidance, encouragement, and support throughout the duration of this work. I am also thankful to Mr. Kunal Vora, Dr.Mr. Rupak Roy and Ms. Shreosee Ghosh for providing the necessary resources and a conducive environment for this work. Their expertise and insights greatly helped shape this project. I extend my heartfelt thanks to all the faculty members and lab staff of the SHRM Biotechnologies Pvt ltd, whose assistance and cooperation have been instrumental. Finally, I am deeply grateful to my family and friends for their constant motivation, patience, and encouragement during this project. PAULAMI CHAKRABORTY ENROLLMENT NO. - 2322211001042 MSC MICROBIOLOGY (2023-2025) 2 LIST OF FIGURES FIGURE NO. DESCRIPTION PAGE NO. 1 Core structure of an Azo dye Page numbers missing? 2 Dye remediation methods comparison 3 Microbial degradation processes 4 Serial dilution of the collected soil 5 Spreading on nutrient agar treated with Methyl Red 6 Sample streaking on nutrient agar plate containing Methyl Red 7 Master plate 8 Streaking on MSM agar containing only carbon source 9 Gram staining 10 Indole test 11 Methyl Red test 12 Voges-Proskauer test 13 Citrate test 14 Starch Hydrolysis test 15 Urease test 16 Gelatinase test 17 Catalase test 18 Oxidase test 19a Sugar fermentation test 19b Dextrose test 19c Lactose test 19d Sucrose test 19e Sorbitol test 19f Maltose test 20 MSA test 21 OD MSM broth taken at 428.87nm at different time intervals 22 pH effect on the degradation ef ciency of Methyl Red dye 23 Temperature effect on the degradation ef ciency of Methyl Red dye 24 Salinity effect on the degradation ef ciency of Methyl Red dye 25 Seeds treated with (a) dye solution at 60°C, (b) dye solution with 0.1gm salinity, (c) basic dye solution, (d) untreated dye solution, (e) water 26 Seed germinated after 7 days (a) dye solution treated at 60°C, (b) dye solution with 0.1 gm salinity, (c) basic dye solution, (d) untreated dye solution, (e) water LIST OF TABLES TABLE NO. DESCRIPTION PAGE NO. 1 Media composition for MSM Page numbers missing? 2 Principle for IMVIC test 3 Media composition for Indole test 4 Media composition of MR-VP broth 5 Media composition for Citrate test 6 Media composition for Strach Hydrolysis 7 Media composition for Urease test 8 Media composition of Gelatinase test 9 Media composition for Sugar test 10 Media composition for MSA ABBREVIATIONS * TCA cycle: Tricarboxylic Acid Cycle * NADH: Nicotinamide adenine dinucleotide-hydrogen * FADH2: Flavin Adenine Dinucleotide * GMO: Genetically Modi ed Organisms * MSM: Minimal Salt Medium * MR test: Methyl Red test * VP test: Voges-Proskauer test * MSA: Mannitol Salt Agar * OD: Optical Density ABSTARCT Synthetic dyes are widely applied in the textile industry because of their color and chemical stability; however, their use raises a lot of environmental concerns. Synthetic dyes are commonly released directly into aquatic and terrestrial

Plagiarized Sources

At the end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential source (if any), generated online using plagiarism checking software is enclosed
https://people.iitism.ac.in/~academics/assets/acad_forms/pg11.pdf

First and foremost, I would like to thank Ms.
https://www.tiktok.com/discover/ rst-and-foremost-i-want-to-thank-me

1 Core structure of an Azo dye Page numbers missing?
https://www.sciencedirect.com/science/article/pii/S027869152300337X

We wish her all the best for her future endeavors.

https://readingswithhope.com/all-the-best-for-your-future-endeavors-messages

* NADH: Nicotinamide adenine dinucleotide-hydrogen * FADH2: Flavin Adenine Dinucleotide * GMO: Genetically Modi ed Organisms * MSM: Minimal Salt Medium * MR test: Methyl Red test * VP test: Voges-Proskauer
https://bio.libretexts.org/Bookshelves/Microbiology/Microbiology_Laboratory_Manual_(Hartline)/01%253A_Labs/1.27%253A_MR-VP_Tests